DNA Damage and Cellular Stress Responses

MGMT Is a Molecular Determinant for Potency of the DNA-EGFR–Combi-Molecule ZRS1

Ying Huang, Zakaria Rachid, and Bertrand J. Jean-Claude

Abstract

To enhance the potency of current EGFR inhibitors, we developed a novel strategy that seeks to confer them an additional DNA damaging function, leading to the design of drugs termed combi-molecules. ZRS1 is a novel combi-molecule that contains an EGFR tyrosine kinase targeting quinazoline arm and a methyltriazene-based DNA damaging one. We examined its effect on human tumor cell lines with varied levels of EGFR and O6-methylguanine DNA methyltransferase (MGMT). ZRS1 was more potent than the clinical methylating agent temozolomide in all cell lines, regardless of their MGMT status. However, its potency was in the same range as or less than that of Iressa, an EGFR inhibitor, against MGMT-proficient cells. In the MGMT-deficient or in MGMT-proficient cells exposed to the MGMT inhibitor O6-benzylguanine, its potency was superior to that of Iressa and temozolomide or a temozolomide + Iressa combination. Cell signaling analysis in A549 (MGMT+) and A427 (MGMT-) showed that ZRS1 strongly inhibited EGFR phosphorylation and related signaling pathways. In addition, the p53 pathway was activated by DNA damage in both cell lines, but apoptosis was significantly more pronounced in A427 cells. Using MGMT shRNA to block endogenous MGMT protein expression in A549 resulted in significant sensitization to ZRS1. Furthermore, transfection of MGMT into A427 greatly decreased the potency of ZRS1. These results conclusively show that MGMT is a critical molecular determinant for the full-blown potency of the dual EGFR-DNA targeting combi-molecule.

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Introduction

EGFR is overexpressed in a large number of cancers including breast, colon and lung cancers, leading to aggressive tumor growth and poor patient prognosis (1–3). Another member of the EGF family of receptors, ErbB2 or Her2, is also frequently overexpressed in various cancer types (4). Ligand stimulation of EGFR results in activation of several downstream signaling pathways, including the RAS/RAF/MAPK and PI3K/Akt pathways. These pathways in turn regulate a variety of other proteins that are involved in cell differentiation, proliferation, motility, and metastasis. Small molecule inhibitors have been designed to inhibit the tyrosine kinase activity of EGFR (5). Two such agents Iressa (AstraZeneca) and Tarceva (OSI Pharmaceuticals) are now in use in the clinical management of lung cancer (6). However, resistance and low response rates to these inhibitors have been observed (5), suggesting that improvement of the potency of the current drugs or the design of new approaches constitutes an urgent need.

Over the past years, we developed a novel tumor targeting approach termed combi-targeting that sought to design molecules designated as combi-molecules to act as an EGFR tyrosine kinase inhibitor and a DNA-damaging agent (7, 8). This has inspired the design of other type of EGFR-DNA targeting molecules (9, 10). One such molecule, ZRS1, contains an EGFR tyrosine kinase targeting quinazoline arm and a methyltriazene-based DNA damaging one. Like its predecessors, under physiologic conditions, ZRS1 is designed to undergo hydrolysis to generate an aminoquinazoline FD105 (an EGFR inhibitor) and a methyl diazonium and an aromatic amine (Fig. 1A). This common methyl diazonium species released by the 2 agents attacks DNA at the O6 or N7 positions of guanine and at the N3 position of adenine.
N7 methylguanine and N3-methyladenine account for 80% of all damage whereas O6-methylguanine accounts for only 6% (12). Despite the greater abundance of N7-methylguanine adducts, the cytotoxic effects of methylating agents are imputed to the O6-methylguanine adducts that generate mismatches with cytosine and thymine. It is believed that the futile attempt by the mismatch repair system to correct these lesions ultimately triggers apoptotic cell death (13). It should be noted that common assays designed to quantitate DNA damage induced by methylating agents require alkaline conditions to generate breaks at N7-methylguanine. The traditional alkaline elution assay (14) that consisted of comparing the rate of elution of DNA from control and treated cells on a polycarbonate filter is now widely replaced by the comet assay. This assay originally described by Olive et al. (15) consists of electrophoresing whole cell nuclei generated in situ on an agarose gel. O6- and N3-methyl adducts are primarily analyzed by liquid chromatography of DNA from cells treated with the radiolabeled alkylating agent.

The O6-methylguanine lesion, which is now widely accepted as the primary cytotoxic lesions induced by

Figure 1. Decomposition of ZRS1 and effect of ZRS1 on EGFR-mediated signaling pathways. A, under physiological condition, the combi-molecule ZRS1 undergoes hydrolysis, releasing a methyldiazonium ion (a DNA methylating species) and FD105 (an inhibitor of EGFR) B, A549 cells were serum starved for 24 hours and subsequently incubated with the indicated concentrations of ZRS1 for 2 hours before stimulation with 50 ng/mL EGF for 15 minutes. C, A427 cells treated as in B.
methylating agent, is efficiently repaired by the DNA repair enzyme O6-methylguanine DNA methyltransferase (MGMT) that removes the methyl adducts from the O6 positions of guanine by transferring it to its internal cysteine residues, resulting in its own inactivation (16, 17). Tumor expression of MGMT varies and correlates with therapeutic response to methytriazene-based chemotherapy in the treatment of glioma (18). It has been shown in many models that tumors expressing MGMT are remarkably resistant to methylating agents. To circumvent this problem, an inhibitor of MGMT termed O6-benzylguanine (BG) has been designed that significantly potentiates the action of temozolomide in xenograft models and is currently under clinical investigation (17, 19).

Recently, in an attempt to increase the potency of methylating agents, we studied a novel strategy that seeks to confer them an EGFR inhibitory arm that can alleviate EGFR-mediated growth and antiapoptotic signaling, thereby sensitizing the cells to the DNA methylating lesions regardless of their MGMT content. Previous studies by our group showed the feasibility of the latter type of EGFR/DNA-directed agents termed combi-molecules and showed that their antiproliferative effect was superior to that of combinations of EGFR- and DNA-targeting molecules (7, 8, 9). However, little is known about the molecular mechanisms regulating the optimal potency of the combi-molecules. In this study, we first examined the molecular events underlying the response to the dual actions of the novel stabilized combi-molecule ZRS1 and subsequently investigated the importance of the cytotoxic O6-methylguanine lesion in its mechanism of action by modulating its direct repair enzyme MGMT. We have chosen to conduct the study in lung cancer cells, a type of tumor in which Iressa and other EGFR inhibitors are most commonly indicated (6). The study focused on the lung cancer cell lines A549 and A427, which were genetically engineered to provide 2 more variants: A427M, its MGMT transfectant, and A549sh in which endogenous expression of MGMT was knocked down. Our hypotheses were strategically tested under isogenic conditions to prevent biases introduced by differences between cell lines. Importantly, the cells were selected on the basis of their p53 wild-type status, which is required for activation of the DNA damage response pathway. It is now well known that in wild-type p53 cells, activation of p53 in response to DNA damage is associated with a rapid increase in its levels, binding to DNA, and transcriptional activation of a number of genes, the products of which trigger cell cycle arrest and apoptosis. One such product is Bax that migrates to the surface of the mitochondria to form pores that allow the release of cytochrome c and subsequent triggering of apoptosis. Thus, the ideal mechanism by which enhanced potency can be triggered by combi-molecules could be one in which it blocks both growth and antiapoptotic signaling mediated by EGFR while activating apoptosis through the p53-activated pathway. Under conditions where the action of the DNA-damaging arm would be mitigated by DNA repair, one would expect the drug to exert potency only through its EGFR-blocking arm. To test this hypothesis, we used the MGMT-expressing A549 lung cancer cells and abrogated MGMT in the latter, both pharmacologically by exposing the cells to O6-benzylguanine or biologically by shRNA technology. Conversely, we used the MGMT-deficient A427 cells in which we challenge our hypothesis by restoring MGMT using gene transfection technology. Our results have shown that in EGFR- and MGMT-proficient cells, despite being able to block EGFR signaling (MAPK and PI3K-AKT pathways), the potency of ZRS1 was comparable or equal to that of the EGFR-inhibitor Iressa. This was associated with its remarkably delayed activation of the p53 pathway that resulted in low levels of apoptotic cell death. By contrast, in MGMT-deficient cells A427 or in MGMT-proficient cells A549 in which MGMT was pharmacologically or biologically suppressed (e.g., A549sh), rapid activation of the DNA damage response and downregulation of the PI3K pathway led to significantly high levels of apoptotic cell death. This is the first report on the optimal conditions for synergistic action of the two mechanistic arms of a methylating combi-triazene.

**Materials and Methods**

**Cell culture**

The human tumor cell lines A549, A427 (lung carcinoma) and HT29 (colon carcinoma), MDA-MB-468 and MDA-MB-231 (breast carcinoma) were obtained from the American Type Culture Collection. The human tumor cell lines HCT116 and HCT116 (p53−/−) (colon carcinoma) were generous gifts from Dr. Janusz Rak from the Montreal Children Hospital (Montreal, Canada). All cell lines were maintained in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% FBS, 10 mmol/L HEPES, 2 mmol/L L-glutamine, and antibiotics (Wisent). All cells were maintained in logarithmic growth and in monolayer culture at 37°C in a humidified atmosphere of 5% CO2 in air.

**Drug treatment**

Iressa was extracted from a tablet provided by AstraZeneca. Temozolomide was extracted from a Temodal tablet from Schering Plough. ZRS1 was synthesized in our laboratory according to known procedures described elsewhere. In all assays, drugs were dissolved in dimethylsulfoxide (DMSO) and further diluted in DMEM media before being added to the cells. The concentration of DMSO never exceeded 0.2% (v/v) during treatment.

**Antibodies and reagents**

Anti-phosphotyrosine, anti-EGFR, anti-p53, anti-GADD45α, anti-MGMT, and donkey anti-goat IgG-HRP antibodies were from Santa Cruz Biotechnology. Anti-phospho-ERK1/2, anti-ERK1/2, anti-phospho-AKT, anti-AKT, anti-phospho-Bad (Ser112), anti-phospho-Bad (Ser136), anti-Bad, anti-Bax antibodies were from Cell
The dose response curve was used to determine 50% cell growth, and the sigmoidal Bio-Rad microplate reader. The results were analyzed by GraphPad Prism (GraphPad Software) and the sigmoidal dose response curve was used to determine 50% cell growth inhibitory concentration (IC50). Each point represents the average of at least 2 independent experiments run in triplicate.

**Western blot analysis**

To evaluate the effect of ZRS1 on EGFR signaling pathway, cells were grown to 80% confluence in 6-well plates, serum starved for 24 hours (DMEM phenol red-free medium without FBS), followed by 2-hour incubation with ZRS1 at the indicated concentrations. Cells were then stimulated with EGF (50 ng/mL) for 20 minutes and collected by scraping in PBS on ice, centrifuged and lysed in ice-cold lysis buffer for 30 minutes (20 mM Tris-HCl pH 7.5, 1% NP-40, 10 mM EDTA, 150 mM NaCl, 20 mM NaF, 1 mM Na3VO4, protease inhibitor cocktail (Roche Molecular Diagnostics)). Lysates were analyzed by western blotting as previously described (8, 9). Membranes were incubated with antibodies coupled with horseradish peroxidase at room temperature for 1 hour. Proteins were visualized by enhanced chemiluminescence (Super West Pico Chemiluminescent Substrate). When the same blot was probed for another protein, the membrane was stripped in Restore Western Blot Stripping Buffer (Pierce) and probed with a secondary primary antibody.

**Alkaline comet assay for quantitation of DNA damage**

The alkaline comet assay was carried out as previously described (7, 8). Comets were visualized at 10× magnification using Leica microscope after staining with SYBR Gold (1:10,000, Molecular Probes) for 45 minutes. DNA damage was quantified using Comet Assay IV software (Perceptive Instruments) and the degree of DNA damage was expressed as a tail moment parameter (i.e., the distance between the barycenter of the head and the tail of the comet multiplied by the percentage of DNA within the tail of the comet; ref. 40). A minimum of 50 comets was analyzed for each drug treatment and the mean tail moments were calculated from at least two independent experiments.

**Generation of stable cell lines**

MGMT plasmid (ORF clone that contains full-length of Homo Sapiens MGMT cDNA) was purchased from OriGene Technologies and transfected into A427, an MGMT-deficient cell line, using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions. Transfected cells were then split at a 1:10 dilution into fresh growth medium 24 hours after transfection and maintained in selective medium containing 750 μg/mL G418 for stable clone selection. The resultant A427 subclone expressing MGMT was named A427M. MGMT-targeted short hairpin RNA (shRNA) vector was purchased from Santa Cruz Biotechnology and transfected into A549, an MGMT-proficient cell line, using Lipofectamine 2000 as described. Transfected cells were maintained similarly as described in selective medium containing 5 μg/mL puromycin for stable clone selection. The resultant A549 subclone was named A549sh. Western blot analysis was used to evaluate MGMT expression.

**Flow cytometry analysis**

To determine cell cycle distribution, cells were grown in 6-well plates and then incubated with drugs for 24 hours. Cells were then harvested, washed in PBS, fixed in cold 70% ethanol, and then stained with 50 μg/mL propidium iodide. Samples were analyzed on a Becton Dickinson Flow Cytometer and cell cycle analysis was done with the ModFit software. To evaluate apoptosis, cells were grown in 6-well plates and then incubated with drugs for 48 hours. Cells were then collected, washed with PBS, centrifuged and subsequently incubated with Annexin V-FITC and propidium iodide using the apoptosis detection kit (BD Bioscience PharMingen). Data were collected using logarithmic amplification of both FL1 (FITC) and FL2 (propidium iodide) channels and quantitated by quadrant analysis of coordinate dot plots.

**Results**

**ZRS1 exhibits the highest potency in an MGMT-deficient cell line**

To examine the growth inhibitory potency of the novel combi-molecule ZRS1, its IC50 values were determined by the sulforhodamine B assay (20) as described in the Materials and Method section in a panel of human cancer cell lines, including the lung cancer cells (A549, A549sh, A427, and A427M), colon cancer [HT29, HCT116, and HCT116 (p53−/−)], and breast cancer (MDA-MB-468 and MDA-MB-231) cell lines. ZRS1 exhibited the highest potency in A427 cells (IC50 = 0.5 μmol/L), an MGMT-deficient cell line (Table 1). In addition, ZRS1 was more potent than the clinical methylating agent temozolomide in all the cell lines tested regardless of their MGMT status (Table 1). However, its potency was in the same range as or less than Iressa, a single-targeted EGFR inhibitor, against MGMT-proficient cells (Table 1). Taking into account that ZRS1 is a combi-molecule with 2 divergent targets, its efficacy was compared with that of a combination of either equi-molar concentration (i.e., combination of the 2 drugs at equal concentration) or equi-effective dose of Iressa and temozolomide in A427 cell line. Our results have shown that the IC50 value of
ZRS1 was 15-fold lower than that of equi-molar and equieffective combinations of Iressa + temozolomide (Table 2), indicating that in the absence of MGMT, ZRS1 can induce stronger potency than Iressa (a monomeric EGFR inhibitor), temozolomide (a monomeric methylation agent), and their corresponding 2-drug combinations.

**ZRS1 inhibits EGFR-mediated signaling pathways**

To elucidate the molecular mechanism underlying the anti-proliferative potency of ZRS1, its effect on EGFR-mediated signal transduction was examined at equi-dose in 2 lung cancer cell lines A549 (MGMT–) and A427 (MGMT–) expressing wild-type p53. The examination was conducted at equi-dose to clearly define at equivalent EGFR inhibitory strength the extent of inhibition of signal transduction in both cells. ZRS1 inhibited the EGFR-stimulated EGFR phosphorylation in a dose-dependent manner at equal strength in both cell lines (Fig. 1B–C). Similarly, this translated into the inhibition of the phosphorylation of 2 major EGFR downstream proteins, ERK1/2 and Akt, in a dose-dependent manner and at almost equal strength in both cells (Fig. 1B–C). Bad is a proapoptotic member of the Bcl-2 family and its phosphorylation leads to the release of the antiapoptotic protein Bcl2 (21, 22). As activation of ERK1/2 and Akt leads to phosphorylation of Bad at serine residues 112 and 136, respectively (21, 22), inhibition of phosphorylation of the latter residues was investigated. As shown in Fig. 1B and C, ZRS1 inhibited the phosphorylation of Bad (S112) and Bad (S136) in a pattern corresponding to the inhibition of EGFR, ERK1/2, and Akt. Inhibition of p-Bad-112 was slightly stronger in the A549 and in A427 cells. Taken together, our results strongly suggest that the quinazoline-based EGFR targeting arm of ZRS1 functions as an EGFR inhibitor and suppresses both the growth signaling and antiapoptotic pathways stimulated by EGFR activation in these cells. Overall, little difference was observed in the strength of inhibition of EGFR-mediated signal transduction by ZRS1 in the 2 cell types, indicating that, as expected, MGMT expression has no significant influence on the action of the EGFR inhibitory arm of the combi-molecule.

**ZRS1 induces DNA damage but differentially affects proteins involved in the DNA damage response in A549 and A427**

To examine whether or not the methyliptiazene-based DNA damaging arm of ZRS1 induces DNA damage, A549 and A427 cells were treated with ZRS1 at various concentrations for 2 hours and then alkaline comet assay was used to evaluate the DNA damage. It should be noted that the purpose of this experiment was to detect the levels of DNA damage induced by ZRS1 in both cell lines. Breaks detected by the comet assay are primarily due to N7-methylguanine under the alkaline condition of the assays, as previously reported by Lacoste et al. (23). The analysis was done 2 hours after drug treatment to minimize the impact of any DNA repair mechanism. In both cell lines, ZRS1 induced significant DNA damage at concentrations as low as 6 μmol/L, and the extent of DNA damage was dose-dependent (Fig. 2A–B). These results indicated that ZRS1 was capable of inducing significant levels of DNA damage in the 2 cell types. Although the latter analysis was

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### Table 1. Antiproliferative effect of ZRS1 on isogenic and established cancer cell lines.

<table>
<thead>
<tr>
<th>Cancer Cell Lines</th>
<th>IC₅₀ (μmol/L) of ZRS1</th>
<th>IC₅₀ (μmol/L) of Iressa</th>
<th>IC₅₀ (μmol/L) of Temozolomide-de</th>
<th>MGMT Status</th>
</tr>
</thead>
<tbody>
<tr>
<td>A549 (lung)</td>
<td>15.6 ± 0.1</td>
<td>18.3 ± 1.6</td>
<td>321.0 ± 10.3</td>
<td>+</td>
</tr>
<tr>
<td>A549sh (lung)</td>
<td>6.8 ± 1.7</td>
<td>17.1 ± 2.1</td>
<td>139.3 ± 23.3</td>
<td>–</td>
</tr>
<tr>
<td>A427 (lung)</td>
<td>0.5 ± 0.0</td>
<td>5.9 ± 0.2</td>
<td>12.4 ± 3.0</td>
<td>–</td>
</tr>
<tr>
<td>A427M (lung)</td>
<td>10.0 ± 1.6</td>
<td>8.2 ± 0.9</td>
<td>348.3 ± 31.5</td>
<td>+</td>
</tr>
<tr>
<td>HT29 (colon)</td>
<td>28.4 ± 2.7</td>
<td>8.6 ± 0.3</td>
<td>630.9 ± 15.3</td>
<td>+</td>
</tr>
<tr>
<td>HCT116 (colon)</td>
<td>24.4 ± 0.9</td>
<td>8.8 ± 0.1</td>
<td>406.9 ± 31.0</td>
<td>+</td>
</tr>
<tr>
<td>HCT116 (p53⁻) (colon)</td>
<td>22.0 ± 1.0</td>
<td>17.0 ± 0.3</td>
<td>422.1 ± 30.2</td>
<td>+</td>
</tr>
<tr>
<td>MDA-MB-468 (breast)</td>
<td>5.2 ± 0.2</td>
<td>4.9 ± 1.0</td>
<td>110.3 ± 25.2</td>
<td>+</td>
</tr>
<tr>
<td>MDA-MB-231 (breast)</td>
<td>39.3 ± 1.8</td>
<td>29.9 ± 0.1</td>
<td>174.9 ± 24.2</td>
<td>+</td>
</tr>
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</table>

(±) MGMT-proficient.
(−) depleted by MGMT-targeted shRNA.
(−) MGMT null.

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### Table 2. Comparison of the efficacy of ZRS1 with the efficacy of Iressa and temozolomide combination in A427 lung cancer cell line.

<table>
<thead>
<tr>
<th>Drugs</th>
<th>IC₅₀ (μmol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ZRS1</td>
<td>0.5 ± 0.0</td>
</tr>
<tr>
<td>Iressa + temozolomide (equal concentration)</td>
<td>7.4 ± 0.4</td>
</tr>
<tr>
<td>Iressa + temozolomide (equal efficacy)</td>
<td>8.1 ± 0.1</td>
</tr>
</tbody>
</table>
done at equi-doses to determine the extent of DNA lesions after a 2-hours drug exposure, due to the marked difference in sensitivity between the 2 cell types to ZRS1 (ZRS1 IC50 growth inhibition, A549–16.6 μmol/L, A427–0.5 μmol/L), the analysis of p53 activation and transactivated proteins was done at equi-effective doses at 4, 24, and 48 hours. In both cell lines, ZRS1-induced accumulation of p53 in a dose-dependent manner (Fig. 2C). However, much lower concentrations of ZRS1 were able to induce more noticeable changes in p53 in A427 (0.5 μmol/L) than in A549 (15 μmol/L). Interestingly, accumulation of p53 did not lead to accumulation of p21 in both cell lines (unpublished data).

Furthermore, we focused our analysis on 2 important proteins that characterize the DNA damage response as Bax and GADD45 (growth arrest and DNA damage-inducible gene; refs. 24–26). Like Bad, Bax is a member of the Bcl-2 family and it promotes apoptosis in response to DNA damage (24, 25). GADD45 is a p53-regulated stress protein that plays an important role in cell cycle checkpoint following exposure to certain types of DNA damaging agents such as UV irradiation and alkylating agents (26). Within 4 hours, ZRS1-induced accumulation of both Bax and GADD45 in A427 in a dose-dependent manner (Fig. 2D), whereas Bax and GADD45 were barely detectable in A549 cells (Fig. 2C). Therefore, the effect of ZRS1 on these proteins was further examined after 24-hour or 48-hour treatment. As shown in Fig. 2, the protein level of Bax became evident only after 48 hours and GADD45 became noticeable after 24 hours in A549. In contrast, the levels of both proteins were induced as early as 4 hours and increased in a dose- and time-dependent manner in A427 cells, indicating that the DNA damage response pathway was more rapidly and
more strongly activated in the sensitive MGMT-deficient A427 cells than in MGMT-proficient A549 and leads to transactivation of growth arrest GADD45 and proapoptotic Bax protein.

ZRS1 induces significantly higher levels of apoptosis in the MGMT-deficient cells

Given that multiple proteins that were affected in response to ZRS1-induced DNA damage play critical roles in cell cycle checkpoint and apoptosis, the effect of ZRS1 on cell cycle distribution and apoptosis was examined in A549 and A427 at equi-doses to assess the strength of the arrest in both cell types. In both cell lines, ZRS1 caused G2/M arrest after 24 hour-treatment (Fig. 3A–B). However, this effect could only be seen at high doses for the MGMT-proficient A549 cells (15–30 μmol/L). In contrast, for the MGMT-deficient A427 cells, cell cycle perturbation was observed at concentrations as low as 0.5 μmol/L, with an S-phase arrest shifting to G2M arrest in a dose-dependent manner. Importantly, ZRS1-induced significant cell death by apoptosis in A427 cells after 48-hour treatment in a dose-dependent manner (Fig. 3C, filled columns). In contrast, within the same 48-hour period, it did not trigger cell death by apoptosis in the MGMT⁺ A549 cells (Fig. 3C, empty columns), indicating that MGMT may play a significant role in blocking the onset of events that lead to apoptosis.

MGMT modulates the efficacy of the DNA damaging arm of ZRS1

To investigate the molecular mechanisms underlying the difference between the efficacy of ZRS1 in A549 and A427, we hypothesize that it may strictly depend on the DNA repair protein MGMT. The growth inhibitory effects of ZRS1 in the absence and presence of O6-benzylguanine were compared in the MGMT-proficient cell lines A549 using the SRB assay. The IC₅₀ value of ZRS1 in A549 was dramatically decreased to 0.7 μmol/L (from 15.6 μmol/L)
and similarly, exposure to O6-benzylguanine considerably decreased the IC50 values of temozolomide in A549 (Fig. 4A and Table 3). However, exposure to O6-benzylguanine did not affect the efficacy of Iressa (Fig. 4A and Table 3). Next, we investigated the molecular events associated with the potentiation of ZRS1 by O6-benzylguanine in A549 by probing the DNA damage response proteins induced following its administration to tumor cells. A549 cells were pretreated with O6-benzylguanine for 12 hours and then exposed to various concentrations of ZRS1 for only 4 hours. As expected, not only the protein level of p53, but also those of Bax and GADD45, increased in a dose-dependent manner after exposure to ZRS1 (Fig. 4A and Table 3), suggesting that the DNA repair activity of MGMT is involved in the delayed activation of the DNA damage response pathway and subsequent triggering of apoptosis.

<table>
<thead>
<tr>
<th>Table 3. A549 cells were treated with 20 μmol/L O6-benzylguanine continuously for 4 days</th>
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<tbody>
<tr>
<td><strong>Drugs</strong></td>
</tr>
<tr>
<td>ZRS1</td>
</tr>
<tr>
<td>Iressa</td>
</tr>
<tr>
<td>Temozolomide</td>
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</table>

The IC50 values were determined by SRB assay and represented as mean ± SEM of at least 2 independent experiments run in triplicate.
The results showed that the IC50 values of both ZRS1 and cell line A427M. Western blot analysis confirmed that MGMT cDNA to express MGMT, leading to the resultant cell line A427 with transfected the MGMT-deficient cell line A427 with the 2 methylating agents ZRS1 and temozolomide. Conversely, we stably transfection enhanced the antiproliferative effect of the 2 methylating agents to inhibit cell growth. suggesting that MGMT expression dampened the ability of temozolomide dramatically increased (Fig. 4C and Table 5), Also, we have shown that the potency of ZRS1 was in the same range or less than that of Iressa, a clinical EGFR-targeting kinase inhibitor, in all cell lines, except in the A427 and the A549sh cells in which MGMT was depleted or absent, ZRS1 was consistently more potent than temozolomide (20-fold to ~26-fold) in all the cells independently of their MGMT status. However, in the presence of MGMT, the growth inhibitory potency of ZRS1 against these cells was comparable or equal to that of Iressa. More importantly, as depicted in Figs. 4D and E, even under conditions where MGMT is depleted or absent, ZRS1 was consistently more potent than temozolomide or Iressa, an indirect indication that when MGMT is absent both the EGFR inhibitory and DNA damaging arm add to the cytotoxic or growth inhibitory potency of ZRS1.

**Table 4. Antiproliferative effects of ZRS1, Iressa, and temozolomide on A549sh cells**

<table>
<thead>
<tr>
<th>Drugs</th>
<th>IC50 (μmol/L) in A427</th>
<th>IC50 (μmol/L) in A549sh</th>
</tr>
</thead>
<tbody>
<tr>
<td>ZRS1</td>
<td>15.6 ± 0.1</td>
<td>6.8 ± 1.7</td>
</tr>
<tr>
<td>Iressa</td>
<td>18.3 ± 1.6</td>
<td>17.1 ± 2.1</td>
</tr>
<tr>
<td>Temozolomide</td>
<td>321.0 ± 10.3</td>
<td>139.3 ± 23.3</td>
</tr>
</tbody>
</table>

The antiproliferative effects of ZRS1, Iressa and Temozolomide on A549sh cells were evaluated by SRB assay and the IC50 values were mean ± SEM of at least 2 independent experiments run in triplicate.

Our results showed that knockdown of MGMT expression enhanced the antiproliferative effect of the 2 methylating agents ZRS1 and temozolomide. Conversely, we stably transfected the MGMT-deficient cell line A427 with MGMT cDNA to express MGMT, leading to the resultant cell line A427M. Western blot analysis confirmed that MGMT was detected in A427M (Fig. 4C and Table 5). The results showed that the IC50 values of both ZRS1 and temozolomide dramatically increased (Fig. 4C and Table 5), suggesting that MGMT expression dampened the ability of the 2 methylating agents to inhibit cell growth.

Finally, we examined the cell cycle distribution and apoptosis induced by ZRS1 in these two stable cell lines. ZRS1 caused G2/M arrest in both cell lines (Fig. 5A–B). However, the arrest was significantly less in the MGMT transfected. In addition, ZRS1 caused stronger G2/M arrest in A549sh than its parental A549 cell line (Figs. 3A and 5A). Similarly, ZRS1 induced stronger G2/M arrest in the MGMT-deficient A427 cell line than its A427M transfectant (Figs. 3B and 5B). Importantly, MGMT transfection suppresses the ability of ZRS1 to induce apoptosis in A427 cells (Fig. 5C, empty columns vs. Fig. 3C, filled columns). Conversely, depletion of MGMT levels by shRNA transfection confers the ability to induce significant levels of apoptosis in A549 cells after 48-hour treatment (Fig. 5C, filled columns vs. Fig. 3C, empty columns).

Experiments in the wild-type, transfected, and shRNA knocked down cells, allowed us to define a growth inhibitory profile for ZRS1, temozolomide and Iressa in the presence or depletion of MGMT in cells with p53 wild-type. As outlined in Figs. 4D and E, ZRS1 was consistently more potent than temozolomide (20-fold to ~26-fold) in all the cells independently of their MGMT status. However, in the presence of MGMT, the growth inhibitory potency of ZRS1 against these cells was comparable or equal to that of Iressa. More importantly, as depicted in Figs. 4D and E, even under conditions where MGMT is depleted or absent, ZRS1 was consistently more potent than temozolomide or Iressa, an indirect indication that when MGMT is absent both the EGFR inhibitory and DNA damaging arm add to the cytotoxic or growth inhibitory potency of ZRS1.

**Table 5. Antiproliferative effects of ZRS1, Iressa, and temozolomide on A427M cells**

<table>
<thead>
<tr>
<th>Drugs</th>
<th>IC50 (μmol/L) in A427</th>
<th>IC50 (μmol/L) in A427M</th>
</tr>
</thead>
<tbody>
<tr>
<td>ZRS1</td>
<td>0.5 ± 0.0</td>
<td>10.0 ± 1.6</td>
</tr>
<tr>
<td>Iressa</td>
<td>5.9 ± 0.2</td>
<td>8.2 ± 0.9</td>
</tr>
<tr>
<td>Temozolomide</td>
<td>12.4 ± 3.0</td>
<td>348.3 ± 31.5</td>
</tr>
</tbody>
</table>

The antiproliferative effects of ZRS1, Iressa and Temozolomide on A427M cells were evaluated by SRB assay and the IC50 values were mean ± SEM of at least 2 independent experiments run in triplicate.

**Discussion**

Over the past few years, we have shown the feasibility of a novel tumor targeting strategy termed combi-targeting that seeks to develop novel drugs termed combi-molecule capable of blocking growth factor-mediated signaling while inducing cytotoxic DNA damage. Combi-molecules such as SMA41 and RB24 have been shown to block EGFR and to methylate DNA (7, 27). Although numerous studies from our group have confirmed the dual actions of combi-molecules (7–9), little is known about the optimal condition under which they exhibit their optimal potency. With the purpose of increasing their stability, we recently reported in a stability optimization study, the synthesis of ZRS1 carrying an acetoxymethyl carbamate moiety that stabilizes the triazene moiety (11). Thus, for this study, we chose to test our hypothesis with ZRS1, a stable and water soluble combi-molecule. We thoroughly investigated herein the molecular mechanisms underlying the dual actions of ZRS1 and attempted to determine conditions under which it can exert its optimal potency.

The ability of ZRS1 to induce DNA damage and to block EGFR phosphorylation in the cells under study was a sine qua non of further mechanistic study. Thus, we first demonstrated that it could induce DNA damage using the comet assay and block EGFR-mediated signaling. Also, we have shown that the potency of ZRS1 was in the same range or less than that of Iressa, a clinical EGFR-targeting kinase inhibitor, in all cell lines, except in the A427 and the A549sh cells in which MGMT was depleted.
The A427 cell line does not express the DNA repair enzyme MGMT and harbors EGFR wild type (28, 29). More importantly, its p53 status is wild type (30), suggesting that its DNA damage response pathway may be intact. Thus we investigated the mechanism underlying its activity in the latter cell line and compared the observed signaling events with those triggered in A549, a methylating agent-resistant lung cancer cell line with a similar

(Fig. 4D-E). The A427 cell line does not express the DNA repair enzyme MGMT and harbors EGFR wild type (28, 29). More importantly, its p53 status is wild type (30), suggesting that its DNA damage response pathway may be intact. Thus we investigated the mechanism underlying its activity in the latter cell line and compared the observed signaling events with those triggered in A549, a methylating agent-resistant lung cancer cell line with a similar

Figure 5. Comparison of the effect of ZRS1 on cell cycle distribution and apoptosis on A549sh and A427M cells. A, A549sh cells incubated in the absence (control) or presence of 15 μmol/L or 30 μmol/L ZRS1, were processed for flow cytometry as described in Materials and Methods. Proportions of cells residing in each cell cycle phase were mean ± SEM of at least 2 independent experiments run in duplicate. B, A427M cells incubated in the absence (control) or presence of 15 μmol/L or 30 μmol/L ZRS1 and cell cycle analyzed as in A. Data represent mean ± SEM of at least 2 independent experiments run in duplicate. C, A549sh cells (filled columns) and A427M cells (empty columns) were incubated with ZRS1 for 48 hours and then analyzed by flow cytometry with Annexin V-FITC/propidium iodide staining to assess cell death. Results were mean ± SEM of at least 2 independent experiments. *, P < 0.05; **, P < 0.01. D, schematic representation of the pathways underlying the optimal potency of ZRS1. O6-MeG represents the O6-methylguanine DNA lesion.
characteristics (e.g., EGFR proficient and p53 wild type status), but expressing MGMT (30-32). Genetic engineering was used to decrease MGMT activity in A549 and increase it in A427. The activity of MGMT in A549 could also be depleted with O6-benzylguanine. Cellular engineering and pharmacologic inhibition have served herein as a powerful platform to resolve a long-standing challenge posed by the dissection of the contribution of individual arms to the overall cytotoxicity of methylating combi-molecules. Although EGF-induced activation of EGFR has now been shown to induce DNA repair enzymes ERCC1, XRCC1, and DNA PK (32, 33), from the current study, it can be inferred that there is no such signaling link between EGFR activation and MGMT activity or expression. ZRS1-inhibited EGFR and Erk1/2 phosphorylation in both cell lines but its activity was significantly reduced in the MGMT-proficient one, indicating that EGFR inhibition was not associated with depletion of MGMT activity or simply that EGFR blockade and DNA damage in the latter cells are not synergistic events for cell-killing. Interestingly, the IC50 values for ZRS1 against A549 cells (15.6 μmol/L) provided a measure of the antiproliferative impact of EGFR blockade, it was in the same range (18 μmol/L) as that of the single-targeted clinical inhibitor Iressa in these cells. More importantly, when A427 cells were transfected with MGMT, the IC50 was brought to the same range as that of Iressa, and conversely when MGMT was pharmacologically abrogated in A549, IC50 values reached the same levels as that against A427 cells (0.5 μmol/L (A427 wild type, 0.7 μmol/L (A549 + O6-benzylguanine); Tables 1 and 2). This convincingly suggests that MGMT is the main abrogator of the potency of the DNA damaging arm or indirectly that O6-methylguanine is the critical cytotoxic adduct that mediates the cytotoxicity of the DNA targeting arm of the combi-molecule. Importantly, one would be tempted to consider the significant potency of ZRS1 under these conditions to be solely due to depletion of MGMT. However, the fact that both pharmacologic and biological abrogation of MGMT in A549 cells did not bring the activity of methylating agent temozolomide to the same levels as ZRS1 in these cells is a strong counterargument (see Fig. 4E). It is also to be noted that the potency of ZRS1 in the absence of MGMT was even stronger than equimolar and equieffective combinations of temozolomide and Iressa (Table 2), suggesting that the subcellular distribution of the combi-molecule could also play a role in its increased potency. Previous studies suggest that the preferential perinuclear distribution of combi-molecules enhanced the levels of DNA damage that they induce (34). The relationship between biodistribution and potency of combi-molecules is discussed elsewhere and is beyond the scope of the current study.

The suppression of EGFR, ERK1/2 and Akt phosphorylation by ZRS1 led to further inhibition of the phosphorylation of downstream protein Bad, indicating that it alleviates the notorious antiapoptotic effect activated through the PI3K pathway (35). Bad is a proapoptotic member of Bcl-2 family. However, survival factors inhibit the apoptotic activity of Bad by phosphorylation at serine 112 and serine 136 (21, 22, 35). Inhibition of phosphorylation at the latter two residues by ZRS1 strongly suggests that it can alleviate antiapoptotic signaling through its EGFR inhibitory arm. More importantly, through the p53-mediated DNA response pathway, ZRS1 induced the proapoptotic protein Bax. By contrast, in A549, where the biological effects of DNA methylation were mitigated by MGMT, Bax expression was dramatically delayed. The mechanism of DNA repair by MGMT is based on its ability to remove the methyl group of O6-methylguanine by transferring onto its own cysteine residue. In the absence of MGMT, the O6-methylguanine lesion persists and attempt to repair it by the mismatch repair (MMR) is known to be responsible for triggering apoptosis (13, 36). The A549 and A427 are MMR-proficient cells (37, 38). By significantly alleviating the EGFR-mediated antiapoptotic signaling, activation of the DNA damage response pathway as indicated by p53, Bax, GADD45 induction, and cell cycle arrest, ZRS1 was able to trigger full-blown apoptosis, leading to submicromolar potency in these cells.

In summary, our study shows that in the presence of MGMT, the growth inhibitory activity of ZRS1 is in the same range as Iressa, with no apparent contribution of the DNA damaging arm. In contrast, as outlined in Fig. 5D, in the absence of MGMT, its ability to not only block the EGFR-MAPK and EGFR-PI3K pathways (Target 1) but also activate p53 pathway and Bax expression (Target 2), translates into significant cell killing by apoptosis. Our current finding provides insight into the optimal conditions under which our methylating combi-molecules exert their potency, namely, intact EGFR pathway, intact DNA damage response, and absence of EGFR-independent DNA repair protein such as MGMT that directly repairs the cytotoxic lesions. This analysis will lead to a more rational development and choice of tumors for which this novel class of drugs can be indicated.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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MGMT Is a Molecular Determinant for Potency of the DNA-EGFR–Combi-Molecule ZRS1

Ying Huang, Zakaria Rachid and Bertrand J. Jean-Claude


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